ASSOCIATIVE OLFACTORY LEARNING IN AGRAULIS VANILLAE (L.) (LEPIDOPTERA, NYMPHALIDAE): BEHAVIORAL AND PHYSIOLOGICAL ASPECTS

By

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Abstract of Dissertation Presented to the Graduate School
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The associative learning capacity along with the morphological and chemical changes in the brain during the acquisition of experience were investigated in male and female nymphalid butterflies, *Agraulis vanillae*. Both males and females were conditioned to chemical stimuli of amyl acetate and butyl acetate, but only males were conditioned to host-plant volatile emissions, although my electroantennogram recordings demonstrate that both sexes of *Agraulis* can detect host-plant aroma as well as both acetates. More females than males were conditioned to amyl and butyl acetates. Female butterflies reared in the laboratory generally exhibited a higher percentage of conditional responses than those collected in the field. The number of conditional responses on the 1st day of experiments was significantly smaller than on the ensuing 2-7 days. Electroantennogram recordings showed that *Agraulis* butterflies respond to the odors emitted from the

abdominal glands of the opposite sex. Odor from virginal females did not elicit electroantennogram response in males.

In the brain of *Agraulis vanillae*, the size of neuropil involved in the processing of olfactory information was found to depend on the butterfly's experience. Butterflies collected in nature have olfactory glomeruli and mushroom body calyces of larger relative size than do butterflies reared and kept in the laboratory in isolation from normal environmental stimuli. The opposite was found for the relative volume of the Kenyon cells region in females, this region being smaller in butterflies collected in the field. No size difference was found in the optic lobes or the central body in either males or females.

Immunocytochemical experiments revealed the presence of the four neurotransmitters: histamine, glutamate, serotonin and GABA in the brain of *Agraulis vanillae*. Capillary Liquid Chromatography showed a difference in the content of another neurotransmitter-candidate, aspartate, between "naïve" and "experienced" females only. It also measured the content of neurotransmitter-candidates: GABA, glutamate, noradrenaline, dopamine, serotonin, taurine and β-alanine. Glutamate, aspartate and taurine were found to have the highest concentration of all the analyzed compounds.

GENERAL INTRODUCTION

In this study I investigate various aspects of morphological, physiological and behavioral phenomena of learning and sensory information processing in the butterfly *Agraulis vanillae*, and present them in the context of the biology of this species.

The butterfly *Agraulis vanillae* is a common species in Florida and in most of the USA. It belongs to the mainly tropical subfamily Heliconiinae of the family Nymphalidae. In Florida this species has several generations a year, and adults and larvae can be collected almost all year around. The host plants of this butterfly are different species of *Passiflora*, *Passiflora incarnata* being the main host plant in Gainesville area. The ubiquity of *Agraulis* and the ease with which it can be reared in the laboratory make this species highly suitable for experimental purposes. What makes this butterfly especially attractive as an object of behavioral and physiological studies is that its adults are long-lived (3-5 weeks) and, as typical of the Heliconiinae in general, possess a very complex behavior. They have highly developed sensory organs, employed in courtship and location of suitable oviposition sites and nectar sources.

In my research I used *Agraulis vanillae* as a model for the study of behavioral, physiological and morphological principles involved in olfaction and learning in Lepidoptera. The existing knowledge of learning and mechanisms of memory formation in insects is largely based on the work on bees and a few other species with well-known complex social communication and behavior. Too few species have been studied with

regard to morphology of the brain regions involved, or suspected to be involved, in processing of sensory information, and the neurophysiological events, which take place in those regions, to fully understand the extent of variability so characteristic of these phenomena in Insecta.

I investigate if *Agraulis vanillae* is capable of associative olfactory learning, and, therefore, if it can adjust its behavior to changes in its environment. The response to these changes, presented to the insect in the form of changing intensities of external stimuli, is the function of insect's brain. This study reports on the effect of the butterfly's individual experience with its changing environment on the morphology and neurophysiology of its brain.

CHAPTER 1
OLFACTORY CONDITIONING OF THE BUTTERFLY *Agraulis vanillae*

Introduction

It is difficult to overestimate the importance of behavioral flexibility to a long-lived animal, which has to respond to multiple stimuli within a complex and variable environment. For example, stimuli associated with food and reproduction may change rapidly throughout a butterfly's life span. Behavioral flexibility may vary considerably between different species and even individuals of the same species. One would expect the specific reproductive success of a long-lived insect that encounters highly variable conditions during its lifetime to hinge upon its individual experience. As a consequence, the ability to successfully cope with problems imposed by a changing environment can be enhanced through associations based on experience. The acquisition of new information through individual reiteration, leading to a specific modification of behavior, is learning (Grier, 1984).

Acquisition and use of new information in insects are well known from studies of honeybee behavior. Learning has been demonstrated by conditioning experiments in which bees were trained to extend their proboscis in response to a presentation of various odors (Frings, 1944; Kuwabara, 1957; Vareschi and Kaissling, 1970; Bitterman et al., 1983; Menzel, 1993; Bitterman, 1996). However, bees are not the only insects capable of learning. Training experiments demonstrate that various insects can learn, including

cockroaches (Gates and Allee, 1933), grain beetles (Cherkashin et al., 1968), fruit flies (Murphy, 1967; Duerr and Quinn, 1982), ants (Hoagland, 1931; Schneirla, 1941), and wasps (Thorpe, 1939; Shafir, 1996). In recent conditioning experiments, two noctuid moth species, *Heliothis virescens* and *Spodoptera littoralis*, acquired a proboscis extension reflex to floral odors, thus demonstrating the capacity to learn (Hartlieb, 1996; Fan et al., 1997).

The preceding discussion shows that learning, or behavioral plasticity, is widespread in the Insecta. For butterflies a few studies have demonstrated that some species use visual cues to associate a host-plant's leaf-shape with its chemical constituents (*Battus philenor* [Papilionidae]; Papaj, 1986) and flower coloration with a nectar reward (*B. philenor*; Weiss, 1997; and *Agraulis vanillae* [Nymphalidae]; Weiss, 1995).

In this study I investigated the learning capability of *Agraulis* by means of classical conditioning experiments, with two floral odors and one host-plant odor as conditional stimuli.

Materials and Methods

Insects

Male and female *Agraulis vanillae* adults and larvae were collected in the Natural Teaching Area near the Department of Entomology and Nematology of the University of Florida, Gainesville, Florida, throughout a period of two years. Larvae were fed in the laboratory on *Passiflora incarnata* (L.), which, among other *Passiflora* species, is the natural local host for *Agraulis*. The plants were obtained every 2 days from the same area where larvae were collected. Although wild adult *Agraulis* were of indeterminate age,

only those having a fresh appearance were used. Hereafter, these will be referred to as "wild" butterflies. Laboratory-reared butterflies were used on the 2nd day after eclosion and will be referred to as "reared".

The main part of the conditioning experiments was done during August-October, 1996 and 1997, during the peak of abundance of *Agraulis* in the area. Additional experiments with host-plant odor were done in September 2000. Experiments were performed in the laboratory (25°C, 65% relative humidity, L:D 16h:8h). Butterflies were kept overnight in the same laboratory room in 25 X 25 X 25 cm screen cages. They were given water every day.

Stimulus Procedures

Three conditional stimuli (CS) were used: amyl acetate (AA), butyl acetate (BA) (both chemicals from Aldrich Chemical Company, Inc., Milwaukee, WI), and the host-plant odor. Ten microliters of AA and 20 microliters of BA were pipetted onto $1cm^2$ Whatman #1 filter papers (additional BA was used to offset the difference in airborne concentration between amyl and butyl acetates due to the difference in their volatility). The host-plant stimulus was a rolled-up 6 cm^2 piece of *Passiflora* leaf that was macerated with forceps. The AA, BA or leaf was inserted in the wide end of a Pasteur pipette, the tip of which was broken to a diameter of 6 mm. The odor was blown over the insect's antennae 5 times by pressing a rubber bulb attached to the pipette.

To account for possible inhibition of the proboscis extension reflex by deterrents emitted by damaged leaf, experiments with direct delivery of host-plant odor from an intact plant were performed. The experimental protocol was as follows. A potted *P. incarnata* plant was placed in an airtight plastic bag. A tube carrying a stream of air,

entering the bag, and a tube leading from the bag to suspended butterflies allowed the delivery of host-plant odor to the experimental site. Control conditioning experiments tested butterflies' response to laboratory air and to the odors emanating from the plastic bag and tubing.

Conditioning Paradigm

The conditioning paradigm developed for moths by Hartlieb (1996) and Fan et al., (1997) was used with modification. During conditioning, butterflies were suspended by the wings on clips, which left the body, feet and head free. After the conditioning trials, the butterflies were returned to their cages. Preliminary experiments determined the most sensitive part of *Agraulis'* body to sugar. For this experiment, the tarsi, the ventral side of both antennae and the proboscis were touched with a pledget of cotton dipped in a 25% sugar solution. The percentage of proboscis extension for each of those sites was calculated. Based on the results of these experiments, the proboscis was chosen as an acceptor of sugar stimulation.

The following conditioning paradigm was employed: (1) presentation of odor (conditional stimulus, CS); (2) application of sugar to proboscis (unconditional stimulus, US); (3) extension of proboscis (unconditional response, UR or conditional response, CR, if elicited by the CS); and, (4) reward (feeding on sugar solution). Immediately after the application of the CS, the US of 25% sugar solution was applied to the proboscis. The insect was allowed to feed on the sugar solution for 5 seconds following the extension of the proboscis. This conditioning procedure was repeated 6 times with each butterfly, after which 4 extinction trials (CS was given without US) followed, except for the 1996 experiments. Extinction trials were performed only on those butterflies, which responded

to CS in no less than half of the conditioning trials (this is why the extinction curve in figures 1, 2 and 3 always starts from a point considerably higher than that of a last trial on the conditioning curves). The intertrial period was 3 minutes. The conditional response (CR) was scored each time when a butterfly extended the proboscis immediately after the presentation of a CS.

To ascertain if butterflies were responding to the odors and not to the mechanical stimulus from the blown air, twice in every conditioning experiment with every butterfly, an empty pipette (without any odor) was used to deliver a clean air stimulus over the antennae. In the control experiments, the CS and US were not paired but were delivered 6 times each in the following sequence: US, CS, CS, US, CS, US, US, CS, US, CS, CS, US with 1.5-min interstimulus intervals. The group of 21 *Agraulis* (12 males and 9 females) was tested on two consecutive days.

The conditioning experiments were repeated every day for 7 days during which time the butterflies remained in good condition. Each butterfly was used for no more than 7 consecutive days. Butterflies were added to the cohort throughout the experiment.

Only butterflies, which did not extend the proboscis in response to the first application of the CS (odor), were used. Unlike analogous earlier experiments with moths (Hartlieb, 1996; Fan et al., 1997), I did not discard the butterflies, which did not react positively to the first application of US (sugar). As my observations demonstrated, all butterflies occasionally failed to extend their proboscis to a touch of sugar, apparently in accordance with their individual physiological state at the moment. Individuals, which did not respond to US, were prompted to taste sugar by having their proboscis uncoiled by an insect pin.

Electroantennogram Recordings

For electroantennograms (EAG), butterflies with wings removed were placed in glass tubes (3cm long, 0.6cm in diameter) with their heads protruding. Glass Ag-AgCl micropipette electrodes filled with 10% NaCl were used. Antennae were placed in the recording electrode, and two other micropipette electrodes filled with 10% NaCl were connected to the butterflies' eyes. Electroantennogram potentials were amplified by a Grass Model P 18 B preamplifier (Grass Instruments Div., Astro-Med, Inc, West Warwick, RI) and displayed on a RD 6110 Omega strip-chart Recorder. Olfactory stimuli were delivered to antennae by blowing air through the glass tube containing either a piece of filter paper with the tested odor on it, or a whole wingless body (or a part of it) of a tested butterfly. A puff of clean air was used as a control.

Statistical Analysis

Data sets of behavioral responses were subjected to analysis of variance (SAS, PROC GLM and options) for each combination of sex, chemical and year to compare the effect of days and trials. The means of days were compared using Duncan's multiple range test and also using contrast statements to compare means of day 1 and the means of the rest of the days.

Results

Preliminary Experiments - Tactile Sensitivity to Sugar Solution

Preliminary experiments were performed to determine which of the three known locations of contact chemosensory sensilla of *Agraulis* would elicit the best response (in the form of proboscis extension) to a touch of sugar solution. A single touch of the sugar-

treated cotton to tarsal sensilla elicited the proboscis extension response from 50.7 ± 2.4% of tested butterflies; to the antenna, 40.5 ± 2.5%; and, to the proboscis, 77.4 ± 2.3%.

Conditioning

There was no proboscis extension to clean air stimuli blown over *Agraulis'* antennae. The control presentation of explicitly unpaired CS and US produced a response in 4.8-9.5% of tested butterflies. Control experiments demonstrated that no conditioning took place with CS-US presented unpaired.

Analysis of variance of the data with appropriate contrast statements tested the hypothesis that the acquisition of conditioning was different between day 1 and the other days. In all conditioning experiments, the data were consistent with a hypothesis that fewer butterflies were conditioned on the first day than on the following days ($p<0.05$).

Amyl Acetate as CS

Amyl acetate (AA) was used because it has a "flowery" smell and I show that *Agraulis* can detect it (see below). Nineteen females and 11 males in 1996, and 6 females and 17 males in 1997 were tested. Generally, more females were conditioned than males with AA as CS. In 1996, there was a statistically verifiable difference between females and males; means ranged between 58-70% for females (Figure 1, a) and 47-63% for

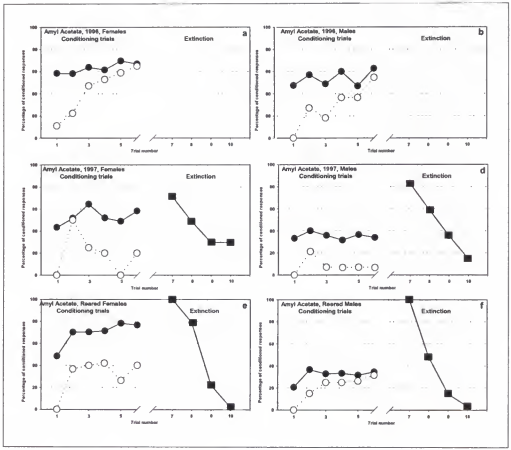

Fig.1 Conditioning of wild and reared *Agraulis* to Amyl Acetate. Open circles-responses to Amyl Acetate on Day 1; filled circles- mean responses on Days 2-7. Filled squares-extinction. Extinction curves correspond to the means of extinction trials on Days 1-7.

males (Figure 1, b) (t=2.6, n=6, $p<0.05$). In 1997, 43-65% of females (Figure1, c) responded, and 32-40% of males (Figure 1, d) (t=5.6, n=6, $p<0.01$). In the group of reared *Agraulis* (20 females, 20 males), females also responded more often than males, 49-78% of females gave the conditional response (Figure 1, e) compared to 21-37% of

males (Figure 1, f). This difference was significant at the $p<0.001$ level ($t=13.9$, $n=6$). The highest level of response achieved in tests with AA as CS was 78% for reared females. The increase in the conditional response during a day of conditioning was recorded for day 1 for wild females and males in 1996 as well as reared males, and for days 2-7 for wild females in 1996 as well as reared females; all significant at $p<0.05$.

Butyl Acetate as CS

Butyl Acetate was chosen as another floral odor to determine if there is any difference in conditioning of *Agraulis* to this compound. With BA as CS, 21 females and 14 males in 1996, and 22 females and 33 males in 1997 were used. As in the experiments with AA as CS, females responded more often than males. In 1996, 38-43% of females (Figure 2, a) and 14-30% of males (Figure 2, b) responded to BA ($t=6.2$, $n=6$, $p<0.01$), and in 1997, 33-54% of females (Figure 2, c) and 34 - 47% of males (Figure2, d) ($t=3.1$, $n=6$, $p<0.05$) responded to BA. For the group of reared *Agraulis* (11 females and 12 males), 48-70% of females responded (Figure 2, e) compared to 34-56% for males (Figure 2, f) ($t=11.2$, $n=6$, $p<0.001$). The highest level of conditioning was attained by reared females (70%). The increase in the conditional response during a day of conditioning was recorded for day 1 in the groups of wild females in 1996 and 1997, as well as reared females, and for days 2-7 in the group of wild males in 1996; all significant at $p<0.05$.

Host-Plant Volatile Emissions as CS

The volatile emissions from host plants were used to ascertain whether or not *Agraulis* could be conditioned to this stimulus. Only 10-16% of females (n=29) (Figure 3,

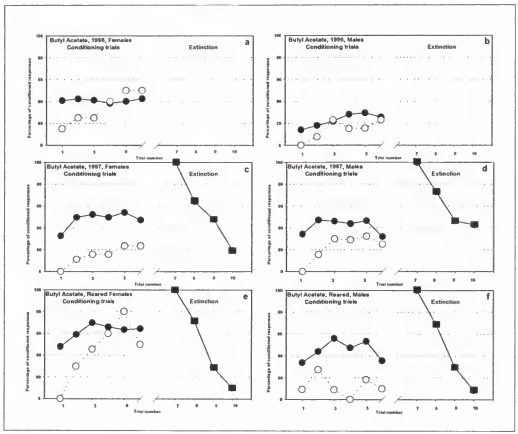

Fig.2 Conditioning of wild and reared *Agraulis* to Butyl Acetate. Open circles-responses to Butyl Acetate on Day 1; filled circles- mean responses on Days 2-7. Filled squares-extinction. Extinction curves correspond to the means of extinction trials on Days 1-7.

a) and 13-19% of males (n=22) (Figure 3, b) were conditioned to this stimulus in the experiments with rolled-up macerated leaf as a source of host-plant odor ($t=6.3$, $n=6$, $p<0.01$). In the additional experiments with a whole *P. incarnata* plant, 22 females and 21 males were tested. This time butterflies demonstrated a better conditioned response,

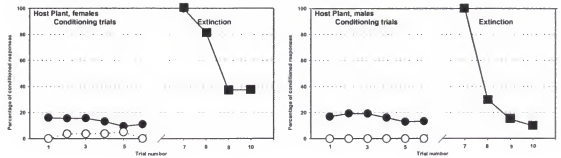

Fig.3 Conditioning of *Agraulis* to Host-plant odor (*Passiflora incarnata*). Open circles-responses to Host-plant on Day 1; filled circles- mean responses on Days 2-7. Filled squares- extinction. Extinction curves correspond to the means of extinction trials on Days 1-7.

with more males responding than females (Fig. 4). Unlike for females (0-9%), the results for males ranged considerably (10-57%) ($t=2.2$, $n=6$, $p< 0.01$).

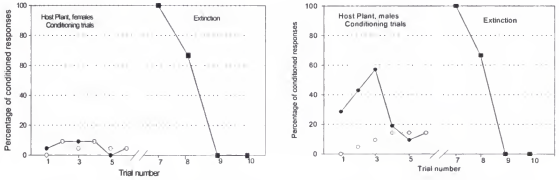

Fig.4 Conditioning of *Agraulis* to Host-plant odor from the whole *Passiflora incarnata* plant. Open circles-responses to Host-plant on Day 1; filled circles- mean responses on Days 2-7. Filled squares- extinction. Extinction curves correspond to the means of extinction trials on Days 1-7.

Electroantennogram Recordings

To determine the sensitivity of *Agraulis* to the chemicals and odors used in the conditioning procedures EAG recording was performed. The response in both males and females to host plant volatiles was equal to the responses to AA and BA. The results are presented in the Figure 5.

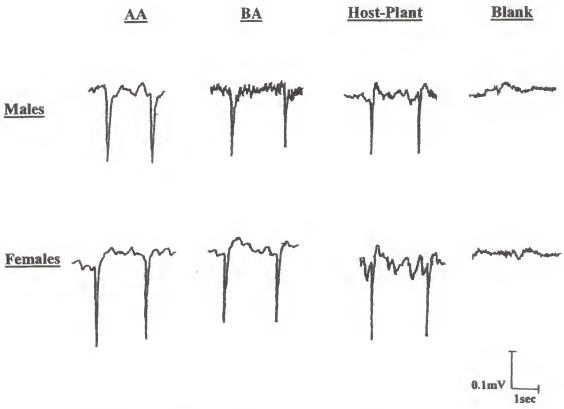

Fig. 5 Electroantennogram recordings from *Agraulis vanillae* with four stimuli- Amyl Acetate (AA) [2µl], Butyl Acetate (BA) [2µl], host-plant odor [6 cm^2 of *Passiflora* leaf, squashed] and a clean air blank.

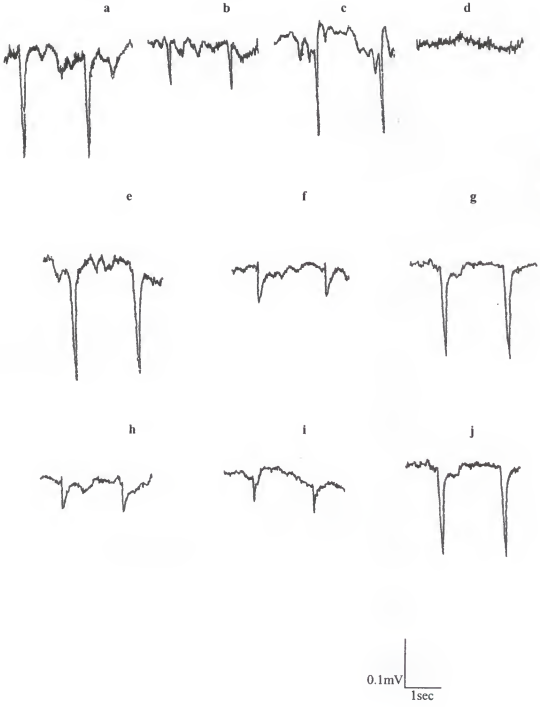

Fig. 6 Electroantennogram recordings from male *Agraulis vanillae* with the following stimuli: **a**- whole male, **b**- same without tip of abdomen, **c**- only tip of male abdomen, **d**- clean air blank, **e**- whole "wild" female, **f**- same without tip of abdomen, **g**- tip of abdomen of the same female, **h**- another whole "wild" female, **i**- virginal female, **j**- 100 µl of hexane solution of female abdomen odors (5 tips of "wild" female abdomens in 10 mL of hexane).

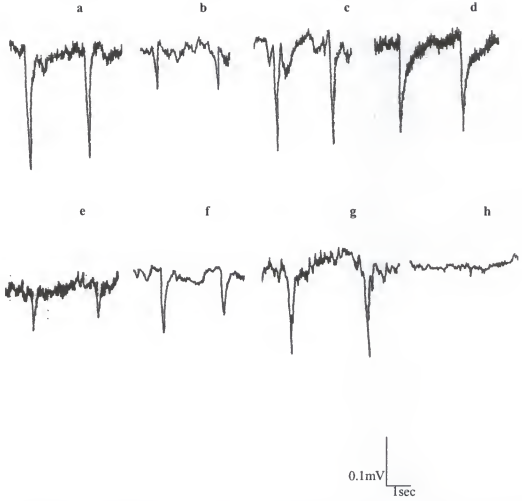

Fig. 7 Electroantennogram recordings from female *Agraulis vanillae* with the following stimuli: **a**- whole male, **b**- same without tip of abdomen, **c**- only tip of male abdomen, **d**- whole "wild" female, **e**- same without tip of abdomen, **f**- tip of abdomen of the same female, **g**-100 µl of hexane solution of male abdomen odors (5 tips of "wild" male abdomens in 10 mL of hexane), **h**- clean air blank.

In the course of EAG recording I undertook to also test the olfaction of *Agraulis* as pertaining to communication between sexes. Butterflies of both sexes responded to the odor of the opposite sex. However, males do not respond to the smell from virginal (reared in isolation in the laboratory) females. From several females, collected in nature

(referred to in the figures as "wild"), usually only two out of four tested with the same male produced in this male an antennogram reaction. Both males and females can respond to the odor of their own sex. However, in females such response is always smaller than their response to the male odor. The source of these odors seems to be located in the tip of abdomen in both males and females. This conclusion is derived at by the observation of the antennograms, obtained for the odors from the whole (wingless) body of the insect, and for those from the abdominal tips only and the remainder of the body (Figs. 6, 7). The tip of the abdomen in both male and female *Agraulis* contains retractable glandular structures of yet unknown morphology and function. These structures are particularly conspicuous in females, where they have a form of two bright orange swellings. Hexane solutions of the chemicals contained therein were also tested, and produced results similar to those for fresh glands (Figs. 6, j; 7, g).

Discussion

These experiments demonstrate that *Agraulis vanillae* associate aromas with an US (touch of sugar). For experiments with insects, the paradigm of classical conditioning is usually employed with the additional step of allowing the insect to feed on the sugar, which is initially applied as US to elicit proboscis extension. This stimulus is given irrespective of the insect's reaction to the CS simply to enhance the performance of the insect. However, if this reinforcer is applied only when the insect's reaction is positive, the animal can associate the CS with the reward, rather than make the desired CS-US association. The association of the CS with a reward is a feature of instrumental, or

operant conditioning. In this study, as in the studies by Bitterman et al. (1983), Hartlieb (1996) and Fan et al. (1997), the sugar was given every time, and not just when the insect responded to the odor. Consequently, the sugar stimulus cannot be associated as a reward by the butterfly. Thus, all these studies are more closely allied with classical than instrumental conditioning.

The insects that have previously been used in conditioning experiments differ in important ways from butterflies. The most important difference between the behavior of butterflies and the other insects is in the significance of odors in their search for food and oviposition sites. Although bees share with butterflies the requisite capacity to locate and feed on nectar sources, they have both a highly developed social behavior and a very well known learning capacity. Moths are in the same order with butterflies and also feed on nectar. Moths, however, perhaps because they fly predominantly under low light conditions or at night, typically have more highly-developed olfactory organs, and probably rely on chemical stimuli more than on visual cues, whereas for diurnally active butterflies the reverse may be true. All of these differences could be responsible, at least in part, for the differences we find between *Agraulis* performance in conditioning tests and that of bees and moths (see below).

Because of these differences, I have introduced a few changes in the generally used "insect" version of the conditioning paradigm. In this study of *Agraulis,* I used only those individuals which did not extend their proboscis to the first application of the CS. Individuals which reacted to CS at once usually continued to do so for the rest of the test. I interpret this behavior as a predisposition of those individual butterflies to the aroma. As a consequence, for such individuals that particular aroma cannot be used as CS, and,

therefore, those individuals were discarded. The percentage of CS-sensitive butterflies was small (1-2%). The percentage of spontaneous response to the CS by *Spodoptera* was higher than for *Agraulis,* 23% for females and 20% for males, yet these moths were retained in the training paradigm (Fan et al., 1997). The small percentage of spontaneous responses to CS in *Agraulis* in my view emphasizes the conclusion that the odors selected to be used as CS were neutral (not innately recognized by *Agraulis*).

Another distinctive feature of *Agraulis* is the individual inconstancy of the proboscis extension reaction to the CS. In the above-cited conditioning experiments with bees and moths, it is not always clearly stated whether or not individual insects remained conditioned once they showed the conditional response for the first time. In my experiments, about 15% of the butterflies always extended their proboscis to CS after having done so once. About the same percentage failed to extend their proboscis even once. In my view, these observations demonstrate that learning in *Agraulis*, as indeed it should be in all animals, depends on the intrinsic associative capacity of individual insects, their behavioral experience, and their physiological condition at the moment.

The highest percentage of conditional response for the average of days 2-7 obtained in my training paradigm was 78% in the group of reared females with AA as CS, although on some days the percentage reached 85-90%. The result for conditioning experiments with BA as CS was somewhat smaller, 70%, and also was obtained for reared females. Unlike reared females, reared males did not respond more often than wild ones. Collectively, females in all the experiments, except the one with Host-Plant as CS, demonstrated greater learning capability than males. It is not clear how this phenomenon can be explained in accordance with male and female behavior. There seems to be no

sign of greater female behavioral complexity, apart perhaps from oviposition site location and host-plant suitability determination by females. Whether or not these are the determining factors for better learning in females remains a question.

The extinction trials demonstrated the resistance to extinction in butterflies that were already conditioned. Often, butterflies whose conditioning was extinguished in 4 trials with the CS not followed by the US responded to the first presentation of the CS on the next experimental day with proboscis extension.

The quantitative nature of the stimulus that was used as CS requires comment. It is important to understand that if the experiment is to explain (at least in some aspects) what happens in nature, the airborne concentration of the stimulus must be relevant to the levels, which can be found in natural conditions. However, purely associative conditioning experiments do not aim to explain the responses to aromas in the way that they apply to behavior in nature. So, for these experiments the airborne concentrations resulting from the dosages of AA, BA and host-plant are much higher than those found in nature.

I decided to determine by EAG recordings whether or not *Agraulis* could detect host plant aroma because initially they failed to become conditioned to it as CS. The same amount of host plant as was used in the conditioning experiments elicited responses comparable to those to AA and BA (Fig. 4). The magnitude of the EAG response to *Passiflora* odor was the same in both males and females. Both sexes may need to respond to host plants. Females need to locate the proper oviposition site and might rely more on the host plant odor than on leaf-shape in their search in densely mixed vegetation. Males, too, may need to recognize the host-plant odor to find the site with newly emerging

females. We do not know how the information about any of these aromas is processed in the central nervous system, but because an insect can detect host plant odor as well as AA or BA, it would seem reasonable that it could be conditioned to all of these stimuli and particularly to the host-plant odor, which is of considerable biological significance. My additional experiments with the host plant odor as CS demonstrated that when *Passiflora* emissions are delivered from an intact plant, 57% of male *Agraulis* show conditioned response, whereas only 9% of females do. It is possible that maceration of the leaf releases certain deterring chemicals, which preclude conditioning to this stimulus of male *Agraulis*. The reason for females to fail to develop a conditioned response to host plant volatiles from an intact *Passiflora* may be contained in the biological significance of host-recognition. Therefore, the decrease or increase in the preference to this odor, or the switch to association of it with feeding rather than with oviposition or mating, could be rendered difficult or even completely impossible for the insect.

Finally, it is especially interesting to compare olfactory conditioning in butterflies to that in moths. First, about as many female *Agraulis* were conditioned as *H. virescens* (Hartlieb, 1996). The highest percentage of CR in male *Agraulis* (63%), for the group tested with AA as CS in 1996, compares to about 58% male *H. virescens*, which were conditioned. Furthermore, the percentage of conditioned *S. littoralis* (Fan et al., 1997) was also similar to the results obtained with *Agraulis*.

This evidence of *Agraulis*' capability of associative learning offers a new direction in the investigation of olfactory interactions between butterflies and their environment. Furthermore, it enables a more fundamental approach to the study of butterfly memory and learning (as discussed in Chapter 2).

The EAG results for *Agraulis*' sensitivity to odors of the opposite sex merit a mention. Field observation of courtship and mating in this species of butterflies does not allow one to see if there is any chemical communication between sexes. The EAG recordings demonstrate that there are sex-specific odors in *Agraulis*, and the employment of these odors is behaviorally regulated. These sex scents are released from the tip of abdomen in both sexes. Emsley (1963) described the anatomy of female scent glands in *Agraulis* and other genera of Heliconiinae. In the genus *Heliconius*, abdominal male scent glands have also been reported (Eltringham, 1925; Crane, 1955).

Nothing is known about the biology of mating in *Agraulis*, but my EAG results suggest that not any female can be attractive to males, and that the information about its attractiveness and suitability as a mate can be conveyed chemically. In *Heliconius* butterflies, for instance, a male transfers certain "antiaphrodisiacs" to the female during mating, thus rendering her repellent to other males and incapable of mating again (Gilbert, 1976). Such a scheme does not seem to fit the situation with *Agraulis*, as virginal females do not possess odors attractive to males, and, also, antennogram response of males to the female sex odor is not negative. It may be, however, that this female odor does not deter male *Agraulis*, but simply arrests its courtship and mating. The other possible explanation would be that females, upon reaching a certain reproductive stage, begin to emit this odor. In such a case, this pheromone is produced by females, and not by males who transfer it to females during mating. The ability of females to respond to male sex odor is harder to explain, as there seems no behavioral support to the notion about females needing to recognize the courting agent. There was no evidence found that females respond differently to sex odors of different males.

Therefore, male sex scent does not seem to carry any information about the individual value of a male as a potential partner.

These results are but the beginning in the investigation of the chemical communication involved in the reproductive biology and behavior of *Agraulis*. Further electrophysiological and ethological experiments are needed to establish the exact nature of sex pheromones and the way, and the behavioral nuances of their employment.

CHAPTER 2
EXPERIENCE-RELATED MORPHOLOGICAL AND CHEMICAL CHANGES IN THE BRAIN OF *Agraulis vanillae*

Introduction

Insect Brain Morphology and Memory Formation

Insect species with complex and flexible behavior possess well-developed proto- and deuto-cerebral regions of the brain, and larger insects have larger brains and more complex histological brain structure and generally exhibit greater complexity of behavior (Goossen, 1949; Bernstein and Bernstein, 1969). The neuropils of particular significance in the processing of information in the insect brain are the mushroom bodies and antennal lobes. Experiments on *Drosophila* (Heisenberg et al., 1985; Han et al., 1992) and *Apis* (Erber et al., 1980; Menzel et al., 1974; Hammer and Menzel, 1998) have shown that the mushroom bodies and antennal neuropil play important roles in olfactory memory formation.

Mushroom bodies of the largest relative size are found in social Hymenoptera. The morphological plasticity of these brain structures has been demonstrated in bees (Withers et al., 1993; Winnington et al., 1996; Robinson, 1998) and ants (Gronenberg et al., 1996). Mushroom bodies increase in size when these insects begin to perform complex and behaviorally more demanding tasks. Neuropil growth related to behavioral changes has also been observed in non-social insects, such as fruit flies and rove beetles (Bieber and Fuldner, 1979; Technau, 1984; Heisenberg et al., 1995). This growth was found to

represent the further arborization and proliferation of existing brain cells, and not the production of new neurons.

Flexibility of behavior and learning have also been demonstrated in different species of Lepidoptera (Swihart and Swihart, 1970; Papaj, 1986; Weiss, 1995, 1997; Hartlieb, 1996; Fan et al., 1997). Butterflies and moths have well-developed mushroom bodies (Sivinsky, 1989; Ali, 1974), and large antennal lobes (Matsumoto and Hildebrand, 1981). Both olfactory and visual learning have been described in *Agraulis vanillae* (Weiss, 1995; Kroutov et al., 1999).

Insect Neurotransmitters and Modulators

In recent years, much has been discovered about chemicals involved in processing of sensory information in the brain of insects. Many neurotransmitters known from vertebrates have been found to play important roles in insect neurophysiology. The best-studied insect neurotransmitters are the biogenic amines serotonin, octopamine, dopamine and histamine, and amino acids GABA (γ-aminobutyric acid), glutamate and taurine. By use of immunocytological and electrophysiological methods, these chemicals have been located in the central nervous system of bees (Bicker, 1993; 1999 a,b; Bornhauser and Meyer, 1996), *Manduca sexta* (Homberg and Hildebrand, 1991; Sun et al., 1993), *Calliphora erythrocephala* (Brotz et al., 1997), *Drosophila* (Bicker, 1991; Nassel, 1999) and a few others.

Small molecules such as histamine or serotonin can act in various ways. They are suited for fast transmission, acting directly as primary transmitters via ligand-gated ion channels. They also could be co-released with other neuroactive substances and thus could modify signals post- and presynaptically.

There is compelling biochemical and electrophysiological evidence that histamine is a neurotransmitter of the depolarizing insect photoreceptors (Bicker, 1999a). Histaminergic neurons were found in photoreceptors of the sphinx moth *Manduca sexta* (Elias and

Evans, 1983), and the flies *Calliphora erithrocephala* and *Musca domestica* (Nassel et al., 1988). Hardie (1989) showed that histamine mediates the action of light on the postsynaptic monopolar cells by gating a chloride conductance. Other experiments of Hardie (1987) demonstrated that only histamine mimics the action of light on secondary neurons. Histamine synthesis was shown to occur in the optic lobes of *Manduca sexta* (Elias and Evans, 1983). Thus histamine fulfils many criteria required for its classification as a neurotransmitter at the photoreceptor synapse of insects (Bornhauser and Meyer, 1997).

Areas of insect midbrain have not yet been extensively analyzed for histamine-immunoreactivity. However, histaminergic neurons have been located in antennal lobes of bees, where all glomeruli were innervated by histamine-containing cells (Bicker, 1999a). Histamine-containing neurons have been detected in mechanosensory cells and their axons in *Drosophila* (Buchner et al., 1993). The presence of histamine-immunoreactive cells in other regions of insect midbrain suggests a more widespread involvement of this chemical as a neurotransmitter or modulator (Bornhauser and Meyer, 1997). In the cockroach *Periplaneta americana*, histamine-immunoreactivity was detected in mushroom bodies, particularly in the calyces, which suggests a possible histaminergic inhibitory control at the input site of the mushroom body (Nassel, 1999).

Histaminergic inhibition occurs at the first synaptic level of the olfactory system of lobsters and is presynaptic- histamine acts on the olfactory receptor cells (Orona et al., 1990). Nassel (1999) suggested that histamine has a similar fast inhibitory action at the earlier stages of synaptic transmission in the olfactory system of some insects.

In insects, GABA-immunoreactivity is predominantly located in local interneurons and to a lesser extent in projection fibers. A high density of GABA-immunreactivity is found throughout the antennal lobe neuropil. Intracellular recordings of bee mushroom body extrinsic neurons have shown that GABA acts as a neuroinhibitory compound

(Michelsen and Braun, 1987). The oscillatory synchronization of projection neurons depends on inhibitory feedback from GABAergic local neurons (Bicker, 1999b). GABAergic neurotransmission appears to be critically involved in olfactory information processing.

Orona (1990) also showed that in lobsters, GABA may act as an inhibitory transmitter at the same synaptic level with histamine, but is likely to act postsynaptically. It was suggested by Nassel (1999), that in insects, similarly to lobsters, both GABA and histamine could be constituents of a dual system of inhibitory transmitters in the antennal lobes.

Physiological experiments suggest that the excitatory transmitter at the neuromuscular junction of insects is glutamate (Jan and Jan, 1976). Immunocytochemical studies (Bicker et al., 1988) confirm that the majority of motorneurons in bees and locusts are glutamate-immunoreactive. Weak glutamate-immunoreactivity was also recorded in the central population of Kenyon cells (Bicker, 1999b).

In both vertebrates and invertebrates, serotoninergic systems share a common characteristic: a small number of serotoninergic neurons innervate a large volume of neuropil. This suggests that serotonin serves a general modulatory role (Nassel et al., 1985). Behavioral studies on bees showed that the proboscis extension reflex to a conditioned stimulus could be suppressed by serotonin, although serotonin does not influence responses to the unconditioned olfactory stimulus (Mercer and Menzel, 1982). These observations suggest a likely modulatory role for serotonin in the antennal lobes (Sun et al., 1993). In *Drosophila* CNS, serotonin was found in outgrowing neurons, which suggests that it may not only act as a neurotransmitter and modulator in neuronal circuits, but may also have additional developmental functions, such as influencing neural outgrowth during insect development (Lundell and Hirsch, 1994).

Taurine is one of the most abundant free amino acids found in insect nervous systems. Its transmitter status is not yet quite clear, but physiological and chemical evidence argues against a role as a classical neurotransmitter and points towards a neuromodúlatory role. It possibly is involved in the neurodevelopment, as it was demonstrated that taurine levels rose 30-fold between pupal and adult stages of the moth *Mamestra configurata* (Bodnaryk, 1981).

Dopamine is another biogenic amine, which is a neurotransmitter candidate. Dopamine-immunoreactive fibers were found in nearly all parts of insect brain, except for the optic lobes (Bicker, 1993; 1999).

The amino acid aspartate is considered a neurotransmitter candidate (Bermudez et al., 1988; Tomlin et al., 1993), but nothing is known about its precise function. In its hyperpolarization effect, it was found similar to glutamate (Hardie, 1987). The work of Ramarao et al. (1987) demonstrated inhibition of glutamate uptake by aspartate in *Drosophila melanogaster*, which indicates that a common carrier mediates the transport of both of these compounds. Another amino acid, β-alanine, as well as the amine noradrenaline, is an inhibitory neurotransmitter in the vertebrate CNS (Van Gennip et al., 1997), but both of these compounds have not yet been reported as neuroactive chemicals for invertebrates.

Some neurotransmitters have been found to be involved in learning and memory formation. The work of Robinson (1998) demonstrated that the brain levels of two amines, dopamine and serotonin, changed during behavioral development of bees. The same research showed that octopamine was present at high levels in the antennal lobes of foragers as compared with nurse bees, regardless of bee's age. It was suggested that octopamine may influence behavioral development of bees by modulating their sensitivity to particular stimuli.

This Work's Objective

Here I studied brain morphology and neurotransmitter content in two groups of *Agraulis*. One group comprised butterflies collected in nature ("experienced" group) and the other group was reared and maintained in the laboratory in isolation from normal environmental stimuli ("naïve" group). In the morphometrical part of the research I investigated the hypotheses that the sizes of brain structures involved in information processing and learning vary according to the individual experience of butterflies, and that such structures should be larger in butterflies exposed to various environmental stimuli than in butterflies deprived of those.

In the immunocytochemical and chromatographic parts of this work I measured the content of brain chemicals potentially involved in processing of sensory information, and tested the hypothesis that the content of these chemicals should be different in the abovementioned groups of *Agraulis*. The compounds I attempted to find, and measure the content of, were GABA, serotonin, histamine, glutamate, aspartate, dopamine, taurine, β-alanine and noradrenaline.

Materials and Methods

Brain Morphometry

Adults and larvae of *Agraulis vanillae* were collected in Gainesville, Florida. Larvae were reared in the laboratory on their natural host-plant *Passiflora incarnata* (L.), picked in the same area where the larvae were found. Laboratory- reared adults spent 48 hours after eclosion in 25X25X25 cm screen cages. The laboratory conditions were- 25°C, 65% relative humidity, L:D 16h:8h. Butterflies were fed a 25% sugar solution.

For the preparation of the histological specimens, butterfly heads were removed and fixed in Bouin's fixative for 2 days. They were then rinsed in 70% ethanol and embedded in paraffin. Heads of 16 reared males, 10 reared females, 17 wild males and 22 wild females were sectioned. The frontal microtome sections were 10 μm thick and were stained with hematoxylin-eosin.

To account for possible shrinkage of the brain tissue in fixative, several brains were kept for 48 hours in Bouin's fixative, and their size was compared to that taken before the fixation. The possibility of tissue shrinkage during the slide preparation procedures was tested by the measurements of the same brains before and after the slide preparation.

To exclude the possible effect of age on the changes in *Agraulis* brain, a control group of 10 males and 10 females, reared in the laboratory, was kept in cages for 20-25 days after eclosion under the same conditions as described for the experimental group. The heads of control butterflies were sectioned, sections stained and brains measured as described above.

Volumetric analysis was performed with an AIS/C image analysis system (Imaging Research, Inc.) interfaced to a Zeiss Axiophot microscope via a Dage 72 CCD camera. The following areas were measured on both sides of the brain: whole brain (protocerebrum, deutocerebrum and tritocerebrum), antennal lobes, olfactory glomeruli, central body, mushroom body calyces, and the regions occupied by Kenyon cells. When areas were measured, this was done without awareness of the group to which that individual belonged. The volume of a brain structure was calculated using the formula

$$Vol_{(object)} = \sum_{i=1}^{n} A_i \times t \times N$$

where A is the area of a measured section, t is the distance between adjacent sections (e.g., section thickness), and N is the number of sections represented by the section A_i. Between 10 and 20 evenly spaced sections were used to determine the volume of each region. The relative volume of each brain structure was calculated as a percentage of the volume of the whole brain.

For statistical analysis of the data, a fixed effects linear model (ANOVA) was fit with PROC GLM (SAS v.8). That is, size was modeled as a function of the fixed effects 'brain region', 'butterfly gender' and 'butterfly group' ("experienced", "naïve" and "control"). All relevant assumptions such as constant variance and normality were formally assessed. Due to the large number of multiple Bonferroni comparisons I tested at the 0.01 level of significance throughout.

Chromatography

Brain samples

The method developed by McKenzie (in press) was used to determine the type and levels of amine-containing neurotransmitters present in butterfly brains. Brains were taken out of live butterflies under a laboratory binocular microscope and their fresh weight was measured on a Mettler AC100 balance. They were then ground in distilled water, 3 brains in 1.5 ml, centrifuged on a Micro-Centrifuge (Model 59A, Fisher) for 2 minutes. The volume of resulting supernatant was brought to 2ml, and the solution was

then filtered through Sterile Acrodisc Syringe Filters (0.2μm). 2μl samples were withdrawn, transferred to microvials and derivatized.

Derivatization procedure

The solutions were stored at room temperature in darkened borosilicate glass vials that had been cleaned using 1 M HCl followed by rinses with HPLC grade water and absolute ethanol (Boyd et al., 2000). Microscale derivatization was performed using a Famos autosampler (LC Packings, San Francisco, CA) to deliver reagents. For derivatization, 0.4 μl 40 mM OPA/50 mM t-BuSH was added to the 2 μl samples, mixed and allowed to react for 5 minutes. Excess thiol was removed by adding 0.4 μl of 1 M IAA and allowed to react for 3 minutes. All samples were derivatized in 250 μl tapered polypropylene microvials that had been pre-cleaned (Boyd et al., 2000).

Capillary liquid chromatography

Capillary LC columns consisted of 50 μm i.d. x 34 cm long fused silica capillaries (Polymicro Technologies, Phoenix, AZ) slurry-packed with 5 μm Alltima C8 particles (Alltech, Deerfield, IL) by a previously described technique (Kennedy et al., 1989). Mobile phase was delivered at 40 μl/min using two high-pressure syringe pumps (100 DM, ISCO, Lincoln, NE) with approximately 90 % of the flow being carried to waste by a splitter thus generating a backpressure of approximately 3500 psi. Injections were performed by the autosampler (Famos), containing a 6-port injection valve (Valco C2) fitted with a 1 μl injection loop. Mobile phase A was 50 mM phosphate buffer pH 6.5 containing 1 mM EDTA while mobile phase B was 35% phosphate buffer and 65% acetonitrile. Mobile phase solutions were degassed prior to loading the syringe pumps by sparging with He for at least 10 minutes.

Electrochemical detection

The working electrode was a carbon fiber microelectrode (9 μm diameter x 1 mm length) fabricated using methods described by Kawagoe et al. (1993). The electrode was inserted using a micropositioner into the outlet of the capillary column mounted in an electrochemical cell containing 0.1 M KCl as supporting electrolyte. Working electrodes were poised at + 0.75 V versus Ag/AgCl reference electrodes. Current was amplified using a Stanford SR-570 low noise current amplifier (Sunnyvale, CA) set at 1 Hz low pass filter. The signal was digitized using a 16-bit AT-MIO data acquisition board (National Instruments, Austin TX) in a 486 DX computer with 5 Hz collection rate.

Statistical analysis

The same statistical analytical procedure as for the "Brain Morphometry" section was employed in this part of the research.

Immunology

For these experiments, paraffin sections of *Agraulis* brains were prepared as described above. I found frozen sections (made on cryostat MICROM HM 505E (Instrumedics Inc.)) unsuited for immunolabeling, as the desired thickness of a section could not have been achieved without a loss of the section's integrity. The antibodies used were as follows:

1) Anti-GABA, developed in rabbit, affinity isolated antigen specific antibody
2) Anti-Histamine, developed in rabbit, affinity isolated antibody
3) Anti-Serotonin, developed in rabbit, delipidized whole antiserum
4) Anti-Glutamate, developed in rabbit, delipidized whole antiserum.

All four antibodies were obtained from SIGMA. The control was normal rabbit serum (SIGMA), diluted at 1:1000 in High Salt Tween buffer (HST).

The immunolabeling procedure employed was as follows. Slides with brain sections (3 groups of 3 sections on each slide) were subjected to an antigen-retrieval: they were placed in a 50-ml beaker with 0.01 sodium citrate buffer pH 6.0, brought to boil, and then kept at 95°C for 10 minutes, and cooled for 15 minutes (Stirling and Graff, 1995). They were then blocked with 2% non-fat dry milk, 1% cold fish gelatin in 1X HST blocking solution for 15 minutes. The primary antibodies, diluted 1:1000 in HST, were applied for 30 minutes. Slides were then washed 3 times, for 5 minutes each time, first in HST, and then twice in phosphate-buffered saline (PBS). The secondary antibody, which was goat serum anti-rabbit, colloidal gold conjugated, diluted 1:5000 in PBS was then applied for 30 minutes, followed by 3 washes in PBS, 5 minutes long each. The slides were then incubated in alkaline phosphatase substrate (Alkaline Phosphatase Substrate kit IV BCIP/NBT, SK-5400 (Vector Laboratories, Inc.) for further 30 minutes. The positive reaction produced dark blue color. The slides, which developed blue color, were washed in water and permanently mounted. They were then studied under Olympus BH2-RFCA microscope. Images from the slides were obtained using a PIXERA PVC 100C camera and PIXERA Studio Pro Software.

Results

Brain Morphometry

Figure 8 shows the sections of the measured brain structures in *Agraulis vanillae*. The protocerebrum is formed by optic lobes (Fig.8, b), mushroom bodies and the neuropils of the central complex, the largest of which is the central body (Fig.8, d). Antennal lobes

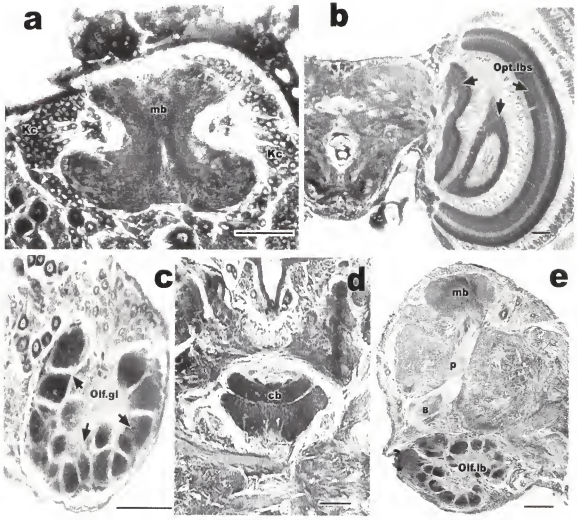

Fig. 8 Sections of the brain of *Agraulis vanillae*. **a:** mushroom body calyx (mb) and Kenyon cells (Kc); **b:** optic lobes (Opt.lbs); **c:** antennal lobe with glomeruli (Olf.gl); **d:** central body (cb); **e:** mushroom body- calyx (mb), pedunculus (p), ß-lobe (B) and antennal lobe (Olf.lb). **a**,**b**,**c**,**d**- frontal sections, **e**-sagittal section. Scale bars- 100µm.

(Fig.8, c, e), composed of olfactory glomeruli and clusters of cell bodies, constitute the deutocerebral part of the brain. The mushroom bodies each consist of a single cup-shaped

calyx and pedunculus (Fig.8, e), which divides into three lobes. The calyx is surrounded by clusters of cell bodies of neurons (Kenyon cells), whose processes comprise the mushroom body (Fig.8, a). Most of these regions exhibit clearly defined boundaries. Because of the absence of a clear boundary between the mushroom body's pedunculus and lobes, and the surrounding diffuse neuropil, only mushroom body calyces were measured.

Whole-brain volume of *Agraulis* showed no significant variation according to group. Volumes were as follows: females -"experienced" $2.28\pm0.24\cdot10^8$ μm^3, "naïve" $2.06\pm0.22\cdot10^8$ μm^3, "control" $2.11\pm0.29\cdot10^8$ μm^3; males-"experienced" $2.25\pm0.08\cdot10^8$ μm^3, "naïve" $2.16\pm0.05\cdot10^8$ μm^3; "control" $2.15\pm0.17\cdot10^8$ μm^3.

There was a significant interaction of gender*group*brain region ($p<0.0001$). Multiple pair-wise comparisons revealed the following patterns: "experienced" individuals of both sexes exhibited significantly larger mushroom bodies and olfactory glomeruli than did "naïve" or "control" individuals (Table 1). The relative volume of mushroom body calyces in "experienced" butterflies was greater than in "naïve" ones by 36% in males, and by 38% in females. Olfactory glomeruli were larger in "experienced" *Agraulis* by 48% in males, and 24% in females.

The Kenyon cells region and antennal lobes showed mixed outcomes. Within the Kenyon cells region, there were no significant differences in volume among the male groups, but "experienced" females exhibited smaller volumes than did "controls". For the antennal lobes, "experienced" males have larger volumes than do "naïve" males. There

Table 1: Relative Volumes of Brain Regions
(values represent percentage of whole brain)

	Mushroom Body Calyx	Olfactory Glomeruli	Kenyon Cells Region	Antennal Lobes	Central Body	Optic Lobes
"experienced" males	2.01± 0.08 ***a***	1.45 ± 0.09 ***a***	0.69 ± 0.07 ***a***	3.74 ± 0.35 ***a***	0.61 ± 0.09 ***a***	64.4 ± 3. 0 ***a***
"naïve" males	1.48 ± 0.05 ***b***	0.98 ± 0.05 ***b***	0.75 ± 0.05 ***a***	3.41 ± 0.10 ***b***	0.68 ± 0.04 ***a***	69.7 ± 8.3 ***a***
"control" males	1.38 ± 0.07 ***b***	0.98 ± 0.09 ***b***	0.72 ± 0.04 ***a***	3.50 ± 0.10 ***ab***	0.66 ± 0.05 ***a***	65.1 ± 2.3 ***a***
"experienced" females	2.18 ± 0.10 ***a***	1.44 ± 0.08 ***a***	0.56 ± 0.03 ***a***	3.90 ± 0.10 ***a***	0.63 ± 0.07 ***a***	61.8 ± 2.6 ***a***
"naïve" females	1.58 ± 0.05 ***b***	1.16 ± 0.05 ***b***	0.73 ± 0.05 ***ab***	4.00 ± 0.20 ***a***	0.64 ± 0.03 ***a***	62.4 ± 2.2 ***a***
"control" females	1.58 ± 0.12 ***b***	1.11 ± 0.04 ***b***	0.84 ± 0.08 ***b***	4.00 ± 0.12 ***a***	0.66 ± 0.02 ***a***	61.1 ± 1.9 ***a***

Relative volumes of brain regions as percentage of the whole brain volume in *Agraulis vanillae*.
Within each box, different small case letters indicate significant differences, whereas
the same letters indicate no difference.

were no differences among the female groups. The central body and optic lobe regions exhibited no significant difference for any pair-wise comparison.

For most brain regions measured, there was no significant difference between male and female volumes. However, the antennal lobes exhibited the following pattern: "naïve" and "control" females have larger antennal lobes than their male counterparts ($p<0.0001$ in each case), but "experienced" females do not differ significantly from "experienced" males.

For all brain regions, the "naïve" and "control" groups exhibited no significant differences in volume, within males or females.

Table 2: Amino acids and amines found in the brain of *Agraulis vanillae*, and their concentration per gram of brain weight (wet).

Noradrenaline	$0.001 \cdot 10^{-2}$ M/g
Dopamine	$0.0004 \cdot 10^{-2}$ M/g
Serotonin	$0.0009 \cdot 10^{-2}$ M/g
Taurine	$0.21 \cdot 10^{-2}$ M/g
β-Alanine	$0.11 \cdot 10^{-2}$ M/g

Chromatography

The content of the neuroactive amino acids and amines in the brain of *Agraulis vanillae* was measured by Capillary Liquid Chromatography. Only for three compounds (aspartate, glutamate and GABA) were the measurements consistent enough to allow statistical comparison among the groups (Fig.9). The only statistically significant

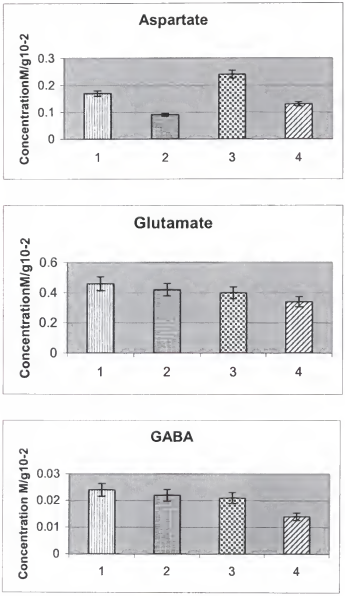

Fig.9 Comparison of the content of three neurotransmitters in the brains of two groups of *Agraulis vanillae*, in $M/g \cdot 10^{-2}$. Vertical line column (1)- "experienced" females, horizontal line column (2)- "naïve" females, checkered column (3)- "experienced" males, diagonal line column (4)- "naïve" males. Bar on each column is an error bar.

difference in the content of tested chemicals was found for aspartate, between "experienced" and "naïve" females ($p<0.001$). Table 2 shows all other compounds, identified in the processed brain samples, and their respective concentrations per gram of the brain weight.

Immunology

Immunocytological experiments showed that the four of the most studied insect neurotransmitters- histamine, GABA, glutamate and serotonin, are also present in the brain of *Agraulis vanillae*. Only histamine-immunoreactivity was observed clearly in the axons of the central brain neuropil (Fig. 10, d, e, f). Histamine-immunoreactive vesicles were found in the Kenyon cells and the cells of the lamina (Fig.10, a, b, c). Serotonin-immuoreactivity (Fig.13) was registered in the form of serotonin-immunoreactive vesicles in all regions of the brain, but could be best observed in the Kenyon cells (Fig.13, d, e). Figure 11 (a-d) shows the GABA-immunoreactivity recorded in the brain of *Agraulis*. It is particularly conspicuous in the Kenyon cells and the mushroom body calyces. Immunotesting for glutamate produced general staining in all regions of the brain (Fig.12), with greater immunoreactivity in the peripheral areas of larger neuropil-optic lobes and mushroom bodies. No sections with antennal lobes were subjected to immunostaining, and consequently, no information was obtained as to the presence of any of the four tested compounds in that very important brain region.

Discussion

Brain Morphometry

The results of my research demonstrate that in *Agraulis*, differences in experience are correlated with changes in the volume of several of its brain regions involved in sensory

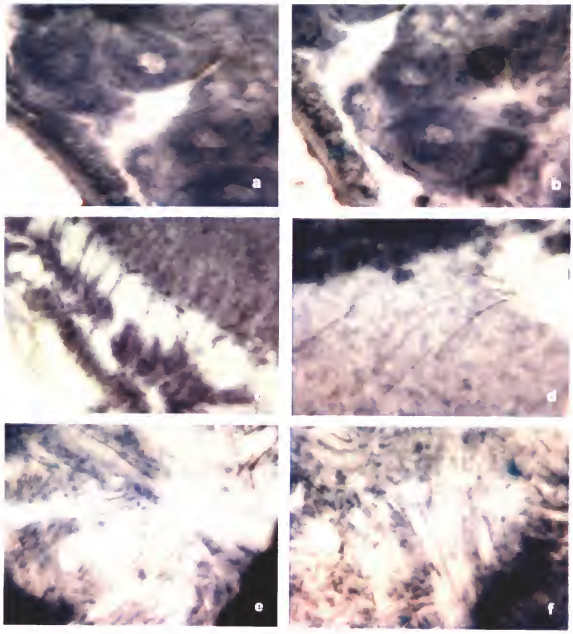

Fig.10 Histamine-immunoreactivity in the brain of *Agraulis vanillae*
a, b- Kenyon cells; **c-** optic cells; **d, e, f-** axons in the central brain. Magnification: **a, b, c, d, e, f-** x482. The immunostaining on the sections can be observed as the dark blue coloration.

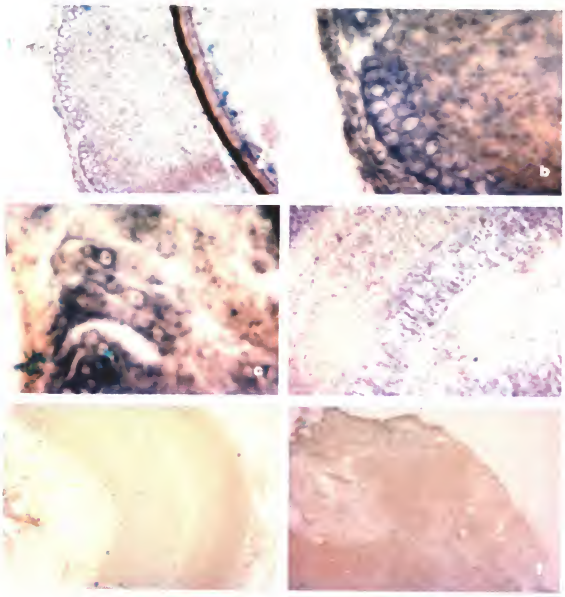

Fig.11 GABA-immunoreactivity in the brain of *Agraulis vanillae*
a, **b**- mushroom body calyx and Kenyon cells; **c**- Kenyon cells; **d**- optic cells; **e**- control, optic lobe; **f**- control, mushroom body calyx. Magnification: **a**, **d**, **e**, **f**- x254; **b**, **c**- x482.
The immunostaining on the sections can be observed as the dark blue coloration.

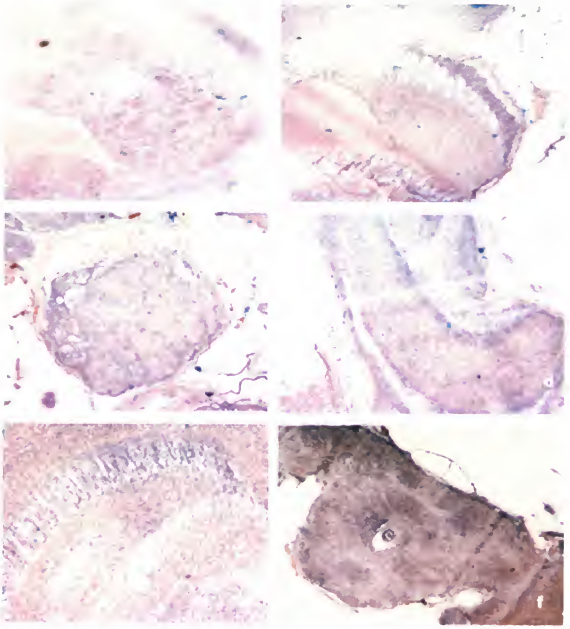

Fig.12 Glutamate-immunoreactivity in the brain of *Agraulis vanillae*
a, **c**, **f**- central brain; **b**, **d**- optic lobes; **e**- optic cells. Magnification: **a**, **b**, **c**, **d**, **e**- x254; **f**- x139. The immunostaining on the sections can be observed as the dark blue coloration.

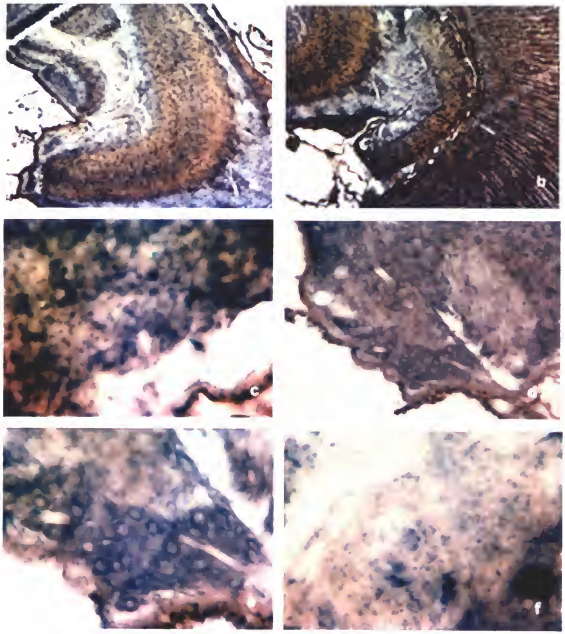

Fig.13 Serotonin-immunoreactivity in the brain of *Agraulis vanillae*
a, **b**- optic lobes; **c**, **f**- central brain neuropil; **d**,**e**- Kenyon cells. Magnification: **a**, **b**- x139; **d**- x254, **c**, **e**, **f**- x482. The immunostaining on the sections can be observed as the dark blue coloration.

information processing and memory formation. During their adult stage (2-4 weeks), *Agraulis vanillae* butterflies must perform various activities, the success of which could be enhanced by learning. Location of feeding sites with flowers that offer sufficient nectar reward, and recognition of potential danger are of importance to both sexes. Female *Agraulis* need to find suitable host-plants on which to lay eggs. This involves not only recognition of the proper host-plant amongst a variety of other plants, but also memory of the location of the host *Passiflora* patch, because butterflies of this species utilize vast habitat ranges and linger at one spot for no longer than is necessary to complete either feeding or egg-laying. Males, in turn, need to locate the host-plant area to encounter females and mate.

Detailed analysis of the captivity conditions and their specific influence on *Agraulis*'s experience, learning and associated morphological changes in its brain was not attempted. However, it seems evident that captive laboratory-reared butterflies would have a greatly reduced range of external stimuli, being deprived of space, visual stimuli and contacts with host-plant, flowers and sex partners.

Generally, measured brain structures were larger (relative to the volume of the whole brain) in "experienced" butterflies than in "naïve" butterflies. But no difference in the relative volume of optic lobes and central body was recorded between the "naïve" and "experienced" groups of *Agraulis*.

The most dramatic increases in relative volume occurred in the mushroom bodies and olfactory glomeruli. This suggests that olfactory stimuli may be of primary importance in driving the structural changes in the *Agraulis* brain. The situation with visual stimuli is not quite clear. Butterflies reputedly rely heavily on visual stimuli (Swihart, 1970; Silberglied, 1979, 1984). As was demonstrated earlier (Weiss, 1995), *Agraulis* is capable of visual as well as olfactory learning. However, no size difference in optic lobes between "experienced" and "naïve" butterflies was observed. It may be that because the optic

lobes are so large (almost equal in size to the central brain), they do not exhibit volumetric change as a result of the difference in experience assessed here.

The relative decrease in volume of the Kenyon cells region is rather hard to explain. However, because there was no change in the whole brain volume, this region's relative decrease could represent an actual compression of the Kenyon cell clusters by the expanding mushroom body calyces. This problem could be addressed by more detailed experimental analysis, for example assessment of cell packing density.

The data presented here are similar in many ways to those of studies in other species of insects, which measured the size differences in brain regions caused by different experience and behavioral repertoire. As in the present study, an increase in the relative volume of mushroom bodies and decrease in relative volume of the Kenyon cells region were reported for ants (Gronenberg et al., 1996) and bees (Withers et al., 1993). Also, there was no increase in the relative volume of the optic lobes in either of these insects. However, unlike in *Agraulis*, in these insects an increase of the whole antennal lobe was found. Olfactory glomerular volume was found to differ between 1-day-old and nurse bees (larger in nurses), but the increase was not maintained in foragers.

For rove beetles, an increase in mushroom body volume and no change in optic lobes volume were recorded (Bieber and Fuldner, 1979). Also, the central body in this insect increased by 73%, as reported in the same study. In fruit flies, growth was recorded in most parts of the brain and was clearly dependent on experience (Heisenberg et al., 1995).

The sexual dimorphism found in the reorganization of some brain structures in *Agraulis*, namely the difference in olfactory glomeruli volume between "naïve" and "experienced" males being twice as great as that in females, can be explained by the differences in behavior of males and females, which may depend on different environmental stimuli. The change in the intensity of these stimuli may affect butterflies

of different sexes differently, thus causing the observed dissimilarity in the brain reconstruction. This dimorphism corresponds with my earlier findings in *Agraulis* learning (Kroutov et al., 1999), where different learning capability was recorded for the two sexes.

Measurements in the control group show that morphological changes in the brain of *Agraulis vanillae* are not age-related, but experience-related, because the relative volumes of the studied brain structures in 2-day ("naïve") and 20-25-day ("control") butterflies were not significantly different. These changes occur in only a few brain compartments that are noted for their role in information processing and learning in insects. This further supports the hypothesis that growth of these brain regions is related to learning experience and behavioral complexity of a butterfly.

The exact cytological events that occur during the reconstruction of insect brain structures are not clear. In different insects they may well be quite different. In Hemimetabolous insects, the growth of mushroom bodies was reported to have its source in the increasing number of Kenyon cells (Rensch, 1956; Cayre et al., 1994). In various Holometabolous insects, however, no production of new Kenyon cells was recorded. Findings in ants suggest that the growth of nerve cell processes leads to an increase in the number of synapses per neuron as the sole cause of the increase in mushroom body size (Gronenberg et al., 1996). The same explanation was suggested for bees (Robinson, 1998), rove beetles (Bieber and Fuldner, 1979) and fruit flies (Heisenberg et al., 1995). This point of view is further supported by the results of studies on *Musca domestica* (Kral and Meinertzhagen, 1989). Apparently, the size and number of synapses in the housefly's lamina depend on the light regime, and they increase with the introduction of brief exposures to light after prolonged periods of darkness.

To elucidate the mechanisms of the observed brain reconstruction in *Agraulis*, neuronal and synaptic density of brain structures would need to be measured. Further

experiments involving the manipulation of various elements of the environment may lead to a better understanding of the exact relation between particular types of information, how they are processed, and changes they cause in the brain of *Agraulis vanillae*. It would be especially interesting to analyze the specific effects of various environmental "deprivations" and, inverted, the effect of additional stimuli on the changes in *Agraulis'* brain structures.

Immunocytology and chromatography

My histochemical and chromatographic experiments revealed the presence of several neuroactive amino acids and amines in the brain of *Agraulis vanillae*. Immunostaining of the brain sections allowed me to locate the immunoreactivity of four neurotransmitter candidates: histamine, serotonin, GABA and glutamate.

My having located histamine-immunoreactivity in the cells of the optic lobe lamina corresponds with existing data on the involvement of histamine in the processing of visual information in insect CNS (Bicker, 1999a). The notion of the greater involvement of histamine in neuroactivity is supported by its broad distribution in *Agraulis* brain, and particularly by its presence in such centers of information processing as mushroom body calyces, where numerous axons have shown histamine-immunoreactivity, and the Kenyon cells. Distribution of histamine-immunoreactivity in the brain of *Agraulis* is very similar to that in the brains of *Gryllus campestris* and *Apis mellifera* (Bornhauser and Meyer, 1997), except for the absence of such reactivity in the mushroom body calyces and Kenyon cells of the latter two insects.

In bees and the moth *Manduca sexta*, serotoninergic neurons were found primarily in antennal lobes (Bicker, 1993; 1999a; Sun et al., 1993). I have no data on any neurochemicals in the antennal lobes of *Agraulis*, as I have not analyzed the sections of this part of the brain histochemically, but I found serotonin-immunoreactive vesicles in

all other regions of the *Agraulis* brain, and most densely they were present in the Kenyon cells.

GABA-immunoreactivity, which in bees is found mainly in the antennal lobes (Bicker, 1993), in *Agraulis* was found in mushroom body calyces and the Kenyon cells. It was also present to a lesser extent in other regions of the brain. The lack of information about the distribution of GABA in *Agraulis* antennal lobes (for explanation, see above) does not allow me to say if GABA-imunoreactivity in the studied butterfly species is indeed the greatest in the area of the mushroom bodies.

Immunostaining of the bee brain for glutamate revealed a high level of glutamate-immunoreactivity in mushroom body calyces (Bicker, 1999b). My experiments show that in *Agraulis*, this immunoreactivity is also located in calyces, particularly in their peripheral zone. A comparable level of glutamate-immunoreactivity was also found in the periphery of optic lobes. Generally, however, immunostaining for glutamate revealed lesser immunoreactivity for this compound than for any of the other three tested compounds.

Simultaneous assay for neuroactive amino acids (aspartate, glutamate, taurine, β-alanine and GABA) and neuroactive amines (noradrenaline, dopamine and serotonin), using Capillary Liquid Chromatography, demonstrated that all of these compounds are present in the brain of *Agraulis vanillae*. Glutamate was found to have the greatest concentration: $0.46 \cdot 10^{-2}$ M/g, aspartate and taurine being next ($0.24 \cdot 10^{-2}$ M/g and $0.21 \cdot 10^{-2}$ M/g, respectively). This result is similar to that obtained for bees, where glutamate and taurine were found to have the highest concentration amongst all of the free amino acids in the brain (Bicker, 1993). Biogenic amines are present in *Agraulis*' brain at much smaller concentrations, the highest concentration being that of noradrenaline ($0.001 \cdot 10^{-2}$ M/g).

Only for three compounds (aspartate, glutamate and GABA) were enough samples tested to compare the content of these chemicals in the two groups of butterflies- "naïve" and "experienced". Only the concentration of aspartate was found to differ significantly between these two groups of *Agraulis*. The great variability of data caused failure in the significance test for other chemicals. This variability can, in my view, be attributed partly to insufficient precision of the chromatographic method employed, as some of the tested compounds (dopamine, GABA) in some runs were not detected at all. This possible lack of precision is due to the fact, that this method is only just being elaborated, and, indeed, these tests were some of the initial few tried.

From these results I conclude that further adaptation of the techniques used in my experiments to the particular purpose of Lepidopteran neurotransmitter study can yield important information on the exact roles played by these chemicals in butterfly learning and in the morphological reconstruction of the brain, which accompanies experience acquisition.

CONCLUSIONS

This study covers important behavioral and physiological aspects of learning in the butterfly *Agraulis vanillae*. It provides evidence as to the learning capability of this species and considers possible implications of such capability in the successful adaptation of the species to its environment. It establishes the connection between the intensity and variety of sensory information available to the butterfly, and the physiology and morphology of the brain regions, which process this information. The following are the most significant conclusions that can be drawn from this work.

- In conditioning experiments, *Agraulis vanillae* can learn to associate aromas with a sugar reward. This finding demonstrates the importance of olfactory information to *Agraulis*, and shows that the basis of the behavioral flexibility of this species is its capability to acquire new reflexes. It indicates how individual experience of the butterfly can affect its behavior through the acquisition of memory.

- In the brain of *Agraulis*, the size of neuropil involved in the processing of sensory information depends on the butterfly's experience. Butterflies, exposed to environmental stimuli of regular intensity and variety, have olfactory glomeruli and mushroom body calyces of larger size than do butterflies kept in isolation from normal environmental stimuli. Both of these brain regions primarily process olfactory information. This emphasizes particular importance of olfactory stimuli in *Agraulis*' adaptation to its

environment, as other regions of its brain do not differ in size between these two groups of butterflies.

- Simultaneous assay by Capillary Liquid Chromatography revealed the presence of several neuroactive amino acids and biogenic amines, known to have neurotransmitter functions in insects. Glutamate, aspartate and taurine have the highest concentration. Other neurotransmitter-candidates (GABA, β-alanine, noradrenaline, dopamine and serotonin) were found, and their content was measured. The concentration of aspartate was higher in "experienced" females than in "naïve" females. This result suggests, that the effect of sensory stimuli on the brain of *Agraulis* is not restricted to the morphological reconstructions of several of its regions, but that it also involves changes in the content of the neuroactive chemicals.

- Immunostaining of the brain sections revealed immunoreactivity of four neurotransmitter compounds (GABA, glutamate, histamine and serotonin) in the brain regions involved in processing of sensory information: mushroom bodies, Kenyon cell regions, optic and antennal lobes. This finding suggests these compounds as being instrumental to neuroactivity in the brain of *Agraulis*, and opens a new direction in the investigation of the fine neurophysiological phenomena underlying information processing and memory formation in Lepidoptera.

- Electroantennogram recordings showed that *Agraulis* butterflies respond to the odors emitted from the abdominal glands of the opposite sex. However, the glands of virginal females do not elicit EAG response in males. This finding demonstrates the presence of an intricate system of pheromonal communication in *Agraulis* and is

additional evidence that butterflies, like moths, use chemical stimuli in intraspecific communication.

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BIOGRAPHICAL SKETCH

Vadim Kroutov was born in Moscow, Russia, on the 30th of April 1971. Having chosen butterflies as an object of a rather serious interest, he entered the Moscow State University (M.S.U.) in the Department of Biology in 1988, and five years later emerged from there a qualified entomologist. His first professional experience was at the Botanical Garden of the M.S.U., where he worked for two blissful years of botanical beatitude, exterminating insect pests. In August 1995 he enrolled in the Ph.D. program in the Department of Entomology and Nematology at the University of Florida, from where he is presently graduating.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas C. Emmel, Chair
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James L. Nation
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Frank Slansky
Professor of Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Marion S. Mayer
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John F. Anderson
Associate Professor of Zoology

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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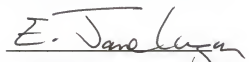

Dean, College of Agricultural and Life Sciences

Dean, Graduate School